Optimization of Microwave-assisted Extraction of Polyphenols from *Enteromorpha prolifra* by Orthogonal Test

LUO Hong-yu[1, 2*], WANG Bin[2*], YU Chun-guang[2], XU Yin-feng[2]

1. School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China
2. School of Food and Pharmacy, Zhejiang Ocean University, Zhoushan 316000, China

Abstract: **Objective** To optimize microwave-assisted extraction of polyphenols from *Enteromorpha prolifra*. **Methods** Based on single-factor tests, an efficient microwave-assisted extraction (MAE) technique was developed to extract bioactive polyphenols from *E. prolifra* through orthogonal $L_{16}(4)^5$ test. **Results** The highest yield (0.923 ± 0.013) mg/g was obtained when microwave power, solvent to raw material ratio, irradiation time, ethanol concentration, and extraction cycles were 500 W, 25 mL/g, 25 min, 40%, and 3, respectively, which was higher than that of Soxhlet extraction with methanol for 6 h, ultrasound-assisted extraction with 40% ethanol for 1 h twice and heat reflux extraction with 40% ethanol for 2 h twice. **Conclusion** This finding indicates that MAE is a superior technique for the extraction of polyphenols due to less impurity, higher time efficiency and yield.

Key words: *Enteromorpha prolifra*; microwave-assisted extraction; orthogonal test; polyphenols
DOI: 10.3969/j.issn.1674-6384.2010.04.011

Introduction

Enteromorpha prolifera (Muell.) J. Agardh belongs to the genus *Caesalpinia* L. of the Ulvaceae family, which is cosmopolitan in distribution and commonly found in a range of shores and habitats. It has a great potential for commercial exploitation because of its abundant and varied chemical composition, quality, and concentration of basic nutriments for other living organisms. Over the past several decades, some active compounds, such as pheophytin, plastocyanin, lectins, and polysaccharides, were isolated from the species (Ambrosio *et al*, 2003; Okai and Higashi-Okai, 1997; Xu *et al*, 2006). However, so far there is no information published on the extraction technology of polyphenols from *E. prolifera*.

At present, several techniques are available for the extraction of polyphenols from plants including Soxhlet extraction (SE) and heat reflux extraction (HRE), but those traditional methods are of low efficiency as they require long processing time and high energy. In order to develop high-efficiency methods, several new techniques were used for extraction of bioactive compounds, such as microwave-assisted extraction (MAE) (Zhang *et al*, 2009; Chen *et al*, 2008; Xu, Yang, and Huang, 2008) and ultrasound-assisted extraction (UAE) (Xu, Zang, and He, 2007). MAE is recommended for its short extraction time, low energy requirement, and high extraction efficiency. Moreover, there are many factors, such as microwave power, irradiation time, composition of solvent, solvent to solid ratio, and extraction cycles, affect extraction efficiency of MAE. Therefore, in this study, we took orthogonal test to optimize the best polyphenol extraction conditions for getting high yield and quality bioactive compounds.

Materials and methods

Plant material, standards, and reagents

The marine alga *E. prolifera* used for this study was freshly collected from the Zhoushan Archipelago coastline of Zhejiang Province, China, in summer of 2008. Samples collected were washed thoroughly with

* Corresponding author: Wang B Tel: +86-580-2555 005 Fax: +86-580-2554 781 E-mail: binwang4159@hotmail.com
Luo HY Tel: +86-580-2555 005 Fax: +86-580-2554 781 E-mail: lisa8919@163.com
Received: April 28, 2010; Revised: August 20, 2010; Accepted:September 15, 2010
Fund: National High Technology Development Project (863 project) (2007AA091701); Zhejiang Provincial Natural Science Foundation (Y2080579); Important Project of Zhejiang Ocean University (21135030107)

fresh water, transported to the laboratory immediately, and dried in the shade at 40 ℃. The shade dried seaweeds were powdered and used for further experiments. The species was identified by Prof. ZHAO Sheng-long at School of Marine Science of Zhejiang Ocean University, where a voucher specimen was deposited (No. ZA0807012).

Folin-Ciocalteu reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid (GA) was purchased from Shanghai Chemical Reagents Co. (Shanghai, China) and was of the highest analytical grade. All other solvents and chemicals were of analytical grade.

Extraction methods

MAE experiments were carried out with an HWC-3LA microwave extraction testing equipment (Beijing Xianghao Equipment Technology Co. Ltd., China). Microwave power (A, 300−700 W), irradiation time (B, 5−40 min), ethanol concentration (C, 10%−60%), ratio of solvent to material (D, 10−40 mL/g), and extraction cycles (E, 1−4) were evaluated for the extraction of polyphenols from *E. prolifera*.

SE was performed in a Soxhlet apparatus. Exhaustive extraction with methanol (85 ℃) was accomplished on 5.0 g drug powder, placed in an extraction bag filter, and impregnated with methanol. Extraction was performed for about 6 h with 150 mL methanol.

HRE was conducted in a water bath at 75 ℃. An amount of 5.0 g drug powder were placed into a 250 mL glass flask with 125 mL 40% ethanol and extracted for two 2 h cycles.

UAE was conducted in an ultrasonic bath (Hechang KH600TDB ultrasonic instrument, Kunshan, China). Drug powder weighing 5.0 g was placed into a 250 mL volumetric flask with 125 mL methanol and sonicated in a water bath at 60 ℃ for three 30 min cycles.

Optimization of polyphenols extraction

An orthogonal $L_{16}(4)^5$ test design was used to investigate the optimal extraction condition of polyphenols from *E. prolifra*. As in Table 1, the extraction experiment was carried out with five factors and four levels. The yield (gallic acid equivalents GAE/seaweed on dry weight basis) of polyphenols was variable-dependent. The polyphenols obtained from the 16 tests were operated following the method above.

Table 1 Factors and levels in orthogonal array design for optimal extraction yield of polyphenols

Levels	Factors				
	A / W	B / min	C / %	D / (mL·g^{-1})	E
1	450	20	25	20	1
2	500	25	30	25	2
3	550	30	35	30	3
4	600	35	40	35	4

Total phenolic content

Total phenols of the extract and fractions were determined according to the Folin–Ciocalteu method described by Duan *et al* (2006) with some modifications. A 1.0 mL aliquot of sample was added to 1.5 mL of deionized water and 0.5 mL of 0.1 mol/L Folin–Ciocalteu reagent, and the contents were mixed thoroughly. After 1 min, 1.0 mL of 20% sodium carbonate solution was added, and the mixture was again mixed thoroughly. The controls contained all the reaction reagents except the sample. After 30 min of incubation at 37 ℃, the absorbance was measured at 750 nm, and compared to a GA calibration curve. Total phenolic content was standardised against GA and expressed as GA in terms of GAE/g seaweed on dry weight basis.

Statistical analysis

All tests were run in triplicate. The average value and standard deviation (SD) were calculated and expressed. All statistic analyses were carried out using Statistic 8.0 software.

Results and discussion

Effect of microwave power on extraction yield of polyphenols

As shown in Fig. 1, the extraction efficiency was improved by raising microwave power from 200 to 500 W, then fell down slowly from 500 to 700 W. Therefore, the best microwave power is 500 W. Overpower may cause the loss of polyphenols.

Effect of ethanol concentration on extraction yield of polyphenols

For MAE, the choice of extraction solvent was taken into account not only for its ability to solve target components but also to absorb microwave energy. Based on our primary tests, the mixtures of water and ethanol were selected for the current study. As shown in Fig. 2, 30% ethanol extraction solvent showed highest yield of polyphenols in 25 min.

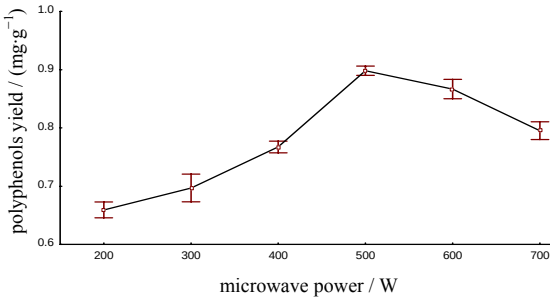

Fig. 1 Effect of microwave power on polyphenols yield

Extraction conditions: 30% ethanol, solvent ratio 25 mL·g^{-1}, irradiation time 25 min, three cycles

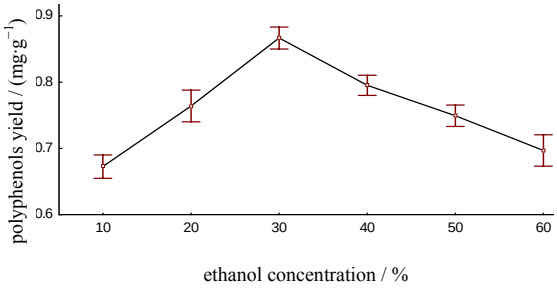

Fig. 2 Effect of ethanol concentration on polyphenols yield

Extraction conditions: microwave power 500 W, solvent to raw material ratio 25 mL·g^{-1}, irradiation time 25 min, three cycles

Effect of irradiation time on extraction yield of polyphenols

The influence of MAE irradiation time on yield of polyphenols was shown in Fig. 3. The yield of polyphenols at the beginning increased along with the prolongation of microwave radiation and reaches its maximum 0.909 mg/g at 25 min and then reduced following the irradiation time prolonged. Over-exposure in the microwave may cause the deterioration of polyphenols. The finding was also observed in the extraction of flavonoids (Xiao, Han, and Shi, 2008) and triterpenoid saponins (Chen, Xie, and Gong, 2007). Therefore, 25 min was chosen as the optimal time point for MAE.

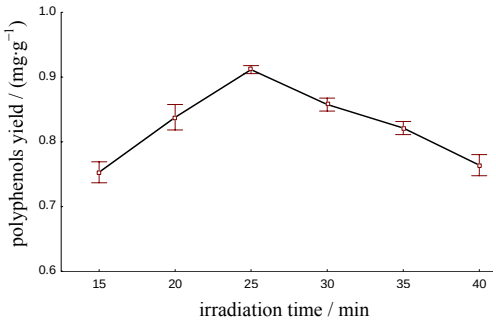

Fig. 3 Effect of irradiation time on polyphenols yield

Extraction conditions: microwave power 500 W, solvent to raw material ratio 25 mL·g^{-1}, ethanol concentration 30%, three cycles

Effect of solvent to material ratio on extraction yield of polyphenols

Generally in conventional extraction techniques a higher volume of solvent will increase the recovery, but in MAE a higher solvent volume may give lower recoveries. It was seen in Fig. 4 that the yield of polyphenols arose along with the increase of solvent to material ratio and reached its maximum 0.912 mg/g at 25 mL/g. It decreased as the ratio was above 25 mL/g. This was probably due to the larger volume of 30% ethanol causing excessive swelling of the material by water and absorbing the effective constituent. Therefore, the value of 25 mL/g was considered as the optimal ratio of solvent to material for the MAE process.

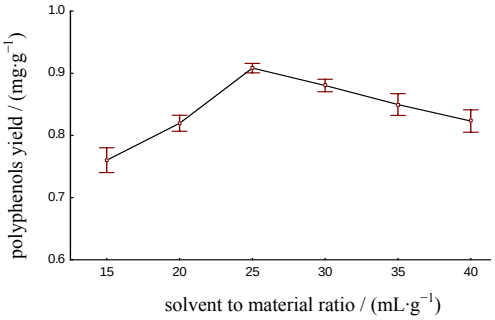

Fig. 4 Effect of solvent to material ratio on polyphenols yield

Extraction conditions: microwave power 500 W, irradiation time 25 min, ethanol concentration 30%, three cycles

Effect of extraction cycles on extraction yield of polyphenols

The yield of polyphenols affected by different extraction cycles was seen in Fig. 5. The yield of polyphenols gets the critical value (0.896 ± 0.025) mg/g when the samples were extracted for three times. And then there is a little increase when extracted exceeds threee times. The yields of two cycles accounted for 91.5% of the yield of six cycles, the third extraction accounted for 5.2% (at 20 mL/g) of the yield of three cycles, and others accounted for 3.3% of the yield of six cycles.

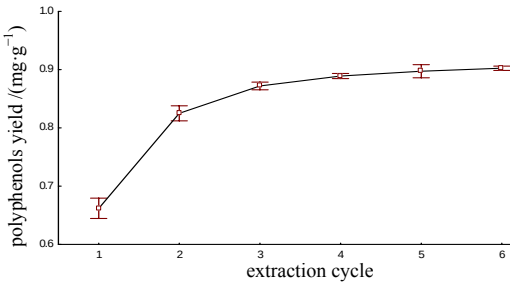

Fig. 5 Effect of extraction cycles on polyphenols yield

Extraction conditions: microwave power 500 W, irradiation time 25 min, ethanol concentration 30%, solvent to raw material ratio 25 mL·g^{-1}

Optimization of extraction parameters of MAE

To the best of our knowledge, various parameters play a great role in the optimization of the experimental conditions for the development of a solvent extraction method. Microwave power, ethanol concentration, irradiation time, solvent to material ratio and extraction cycles are generally considered to be the most important factors that affect the yield of MAE. The investigated levels of each factor were selected depending on the above results of the single-factor. In the present study, all selected factors were examined using an $L_{16}(4)^5$ orthogonal test design. The analysis results of orthogonal test are presented in Table 2. Although the maximum yield of MAE was (0.906 ± 0.008) mg/g, we cannot choose the corresponding extraction conditions as the best technique. In view of orthogonal analysis, we applied statistical software to calculate the values of *K* and *R*. The factors that influenced the yield of MAE were listed in a decreasing order as follows: E > A > D > B > C according to the *R* value. Thus, the maximum yield of the polyphenols was obtained when extraction cycles, microwave power, solvent to material ratio, irradiation time, and ethanol concentration were $E_3A_2D_2B_2C_4$ (3 times, 500 W, 25 mL/g, 25 min, and 40%), respectively. According to the *R* value, we could find the extraction cycle was the most important determinant of the yield of MAE and the level of ethanol concentration was the minimal impact factor in the experiment. We obtained the high yield and quality bioactive polyphenols through confirmatory test, with a yield of (0.923 ± 0.013) mg/g.

Table 2 Analysis of $L_{16}(4)^5$ test results (*n* = 3)

No.	A / W	B / min	C	D / (mL·g^{-1})	E	Yield of polyphenols[b]/g
1	450	20	25%	20	1	0.655 ± 0.036
2	450	25	30%	25	2	0.864 ± 0.017
3	450	30	35%	30	3	0.889 ± 0.014
4	450	35	40%	35	4	0.877 ± 0.026
5	500	20	30%	30	4	0.893 ± 0.019
6	500	25	25%	35	3	0.906 ± 0.008
7	500	30	40%	20	2	0.853 ± 0.042
8	500	35	35%	25	1	0.742 ± 0.028
9	550	20	35%	35	2	0.825 ± 0.015
10	550	25	40%	30	1	0.708 ± 0.034
11	550	30	25%	25	4	0.894 ± 0.016
12	550	35	30%	20	3	0.887 ± 0.038
13	600	20	40%	25	3	0.874 ± 0.027
14	600	25	35%	20	4	0.887 ± 0.017
15	600	30	30%	35	1	0.643 ± 0.019
16	600	35	25%	30	2	0.792 ± 0.043
K_1	3.285	3.247	3.247	3.282	2.748	ΣY=13.189
K_2	3.394	3.365	3.287	3.374	3.334	
K_3	3.314	3.279	3.343	3.282	3.556	
K_4	3.196	3.298	3.312	3.251	3.551	
R[a]	0.198	0.118	0.096	0.123	0.808	

a: result of extreme analysis

b: yield of polyphenols as mg GAE /g seaweed on dry weight basis

Comparison of MAE with SE, HRE, and UAE

SE, HRE, and UAE were the most common methods for the extraction of bioactive components from natural products. It can be seen in Table 3 that crude extract yield of SE is maximum among the four compared methods and MAE is the second highest yield method. Though the crude extract yield was slightly lower than that of SE, MAE took only one by 4.8 times of SE and the extraction solvent (40% ethanol) was much safer than methanol used in SE. Polyphenols yield of MAE, a more important index than crude extract yield, was higher than other three methods, which suggested that MAE was more suitable for the extraction of polyphenols due to the little impurity in the crude extract. Given the above data, MAE has been suggested to be a superior alternative technique to SE, UAE, and HRE in the practical production of algae polyphenols because of less impurity, higher time efficiency and yield of polyphenols.

Conclusion

In the present study, an efficient MAE process has been developed for fast extraction of polyphenols from *E. prolifra* using single factor experiments and orthogonal test design. The yield of polyphenols under

Table 3 Comparison of MAE with other extraction methods (*n* = 3)

Extraction methods	Extraction time	Solvent	Solvent consumption / (mL·g^{-1})	Yield of crude extract [a]/g	Yield of polyphenols [a]/g
SE	6 h	methanol	25	8.32 ± 0.35	0.889 ± 0.031
UAE	1 h × 2	40% ethanol	25	6.27 ± 0.23	0.792 ± 0.038
HRE	2 h × 2	40% ethanol	25	7.14 ± 0.43	0.836 ± 0.021
MAE	25 min × 3	40% ethanol	25	8.17 ± 0.19	0.923 ± 0.013

a: yield of polyphenols as mg GAE/g seaweed on dry weight basis

the optimal extraction condition (microwave power 500 W, ethanol concentration 40%, irradiation time 25 min, solvent to material ratio 25 mL/g, and extraction for 3 times) was (0.923 ± 0.013) mg/g. Compared with conventional extraction methods employed in this study, the main advantages of the MAE procedure are low consumption of organic solvent, and particularly, the rapid extraction, which was performed in only 25 min, achieving relatively higher yield for polyphenols. Further efforts are underway to isolate and identify the active phenolic compounds from the alga.

On the basis of these considerations, the optimized MAE method should be favoured for the routine screening analysis of polyphenols from seaweeds. The potential use of MAE for the efficient extraction of natural products may assist in expediting the chemical analysis and characterization of the biological activities of such compounds. With all these merits, MAE should be considered for wider application in the extraction and purification of polyphenols from plants.

References

Ambrosio AL, Sanz L, Sánchez EI, Wolfenstein-Todel C, Calvete JJ, 2003. Isolation of two novel mannan, and *L*-fucose-binding lectins from the green alga *Enteromorpha prolifera*: Biochemical characterization of EPL-2. *Arch Biochem Biophys* 415(2): 245-250.

Chen LG, Jin HY, Ding L, Zhang HR, Li J, Qu CL, Zhang HQ, 2008. Dynamic microwave-assisted extraction of flavonoids from *Herba Epimedii*. *Sep Purif Technol* 59(1): 50-57.

Chen Y, Xie MY, Gong XF, 2007. Microwave-assisted extraction used for the isolation of total triterpenoid saponins from *Ganoderma atrum*. *J Food Eng* 81(1): 162-170.

Duan XJ, Zhang WW, Li XM, Wang BG, 2006. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. *Food Chem* 95(1): 37-43.

Okai Y, Higashi-Okai K, 1997. Potent anti-inflammatory activity of pheophytin a derived from edible green alga, *Enteromorpha prolifera* (Sujiao-nori). *Int J lmmunopharmac* 19(6): 355-358.

Xiao WH, Han LJ, Shi B, 2008. Microwave-assisted extraction of flavonoids from *Radix Astragali*. *Sep Purif Technol* 62(3): 614-618.

Xu DL, Huang XC, Ou CR, Yang WG., Wang HH, 2006. Effects of polysaccharide from *Alga Entermorpha* prolifera on activities of superoxide dismutase (SOD) and lysozyme (LSZ) in haemolymph of *Scallop chlamys nobilis*. *Fish Sci* 25(2): 72-74.

Xu H, Zang Y, He C, 2007. Ultrasonically assisted extraction of isoflavones from stem of *Pueraria Zobata* (Willd.) Ohwi and its mathematical model. *Chin J Chem Eng* 15(6): 861-867.

Xu HY, Yang YF, Huang CY, 2008. Study of microwave-assisted extraction of polysaccharide from *Radix Astragall*. *Chin Tradit Herb Drugs* 39(10): 1496-1499.

Zhang P, Liao ZG, Liang XL, Zhao GW, 2009. Microwave-assisted extraction of Shuxiong Tablets prescription by uniform design. *Chin Tradit Herb Drugs* 40(1): 45-49.